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Nutritional Modulations Used to Translate a Rabbit Model of Atherosclerosis — A Systematic Review and Meta-analysis

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Abstract

Dietary cholesterol has been suggested as a cause of dyslipidemic atherosclerosis with scarce convincing evidence. A systematic review and a meta-analysis were conducted in MEDLINE (2004–2015) to screen randomized controlled trials (RCTs) that used cholesterol-fed rabbits as a model of atherosclerosis. A total of 32 RCTs ($n = 1104$ New Zealand rabbits; 4.37 ± 2.52 months old) reported lipid and lipoprotein outcomes following cholesterol intake ($0.98 \pm 0.67\%$) for a duration of 8.90 ± 7.26 weeks. Cholesterol intakes significantly raised combined lipid and lipoprotein outcomes (standardized mean difference) in a random-effect model by 5.618 (95% CI: 4.592, 6.644; $P = 0.0001$). The value of I^2 , heterogeneity, was 89.387%, indicating real variation. A subgroup analysis based on the duration and amount of cholesterol feeding in a mixed-effects analysis showed combined heterogeneous effects of 2.788 (95% CI: 2.333, 3.244; $P = 0.000$; $Q = 112.206$; $df = 14$) and 5.538 (95% CI: 4.613, 6.463; $P = 0.000$; $Q = 31.622$; $df = 6$), respectively. Random-effect meta-regression conducted using cholesterol moderator did not support causal effects of dietary cholesterol in inducing atherosclerosis, which may be due to significant publication bias. These high levels of heterogeneity among studies may decline fidelity of this animal model for translation of dyslipidemic atherosclerosis.

Keywords: Atherosclerosis, rabbits, cholesterol, lipoproteins, meta-analysis

1. Introduction

Atherosclerosis is a disease that belongs to both antiquity and modern era [1]. This economically important disease is an outcome of both genetic and environmental risk factors and their interactions [2]. Inflammatory and metabolic derangements and their synergy are the main

causes in the etiopathogenesis of atherosclerosis [3]. However, all aspects of atherosclerosis are not known at the present time, and more human and animal studies are requested to decode the black box of atherosclerosis. Since we are not able to do interventional studies in humans, animal models are good simulated and translated tools in this endeavor.

Many seminal reviews (e.g., see [4–6]) have shown that rabbits are good, reliable, and cheap animal models of atherosclerosis, with a high degree of comparability to human's atherosclerosis. In this context, low-density lipoprotein cholesterol (LDL-C) is predominant plasma lipoprotein in rabbits and humans [6].

Despite these data, the amount of dietary cholesterol, the diet formula, and the duration of cholesterol feeding necessary to induce translated atherosclerosis are not conclusive among experimental studies (e.g., see [7,8]). In this line, Prof. Watanabe and colleagues [9] partially solved this problem by producing a genetic rabbit model of hypercholesterolemia many years ago. However, this expensive model is not globally available in all laboratories and is not suitable for translating nutritional interventions that lead to atherosclerosis. Watanabe rabbit is still not a pet animal model for studying atherosclerosis in all laboratories.

In addition, there is not any concert about the methodology used for inducing atherosclerosis in nongenetic animal models like New Zealand rabbits (NZRs) among various studies. In this sense, reliable and loyal translation of atherosclerosis in rabbits were hampered by methodological limitations, including suitable sample size, suboptimal biomarker assay, amount of cholesterol intake, routes of cholesterol intake, and duration of cholesterol intake, among others. The aforementioned limitations result in low statistical power to translate atherosclerosis in animal models like rabbits.

To obtain a more truthful and defined estimation of association between cholesterol intake and atherosclerosis (i.e., the occurrence of dyslipidemia), to do root cause analysis, and to explore the source of heterogeneity among randomized controlled trials (RCTs), we conducted a meta-analysis of RCTs by evaluating the associations of dietary cholesterol with the dyslipidemia component of atherosclerosis in rabbits.

2. Materials and methods

2.1. Study selection

A comprehensive electronic search of the MEDLINE (National Library of Medicine) database was conducted to identify English-written studies published between January 1, 2004, and January 1, 2015, using ("Atherosclerosis"[Mesh]) AND "Rabbits"[Mesh] AND (Animals[Mesh:noexp]) in PubMed as Medical Subject Headings (MeSH). Two investigators (IK and SSM) coincidentally assessed titles and abstracts of articles generated by the literature research after removal of duplicated records. Discrepancies in opinion between the two investigators were adjudicated by a third investigator (GB). We considered the following

criteria in the selected studies for meta-analysis: (1) inclusion of cholesterol in diet was the main part of the dietary intervention; (2) comparison groups could include a control diet or an intervention in which cholesterol intake was quantitatively different from cholesterol intake in the experimental groups; (3) among pharmacological studies, only results of the control group and the cholesterol-fed group were selected, and the rest of the drug-treated groups were excluded; (4) we included studies that reported net quantity of lipid and lipoprotein profiles and studies that reported changes graphically have been excluded; (5) studies that solely considered other end points such as inflammatory end points of atherosclerosis, histopathologic descriptive end points, and vascular imaging of atherosclerosis were excluded; (6) all studies that reported dyslipidemia in genetic engineered models and strains were excluded; and (7) all studies that focused on lipid, and lipid profiles of cholesterol-fed New Zealand rabbits (NZRs) were included to do meta-analysis over their results. In this continuum, 32 articles met the aforementioned eligibility criteria for inclusion in meta-analysis [10–41]. Figure 1 summarizes information on the study selection process.

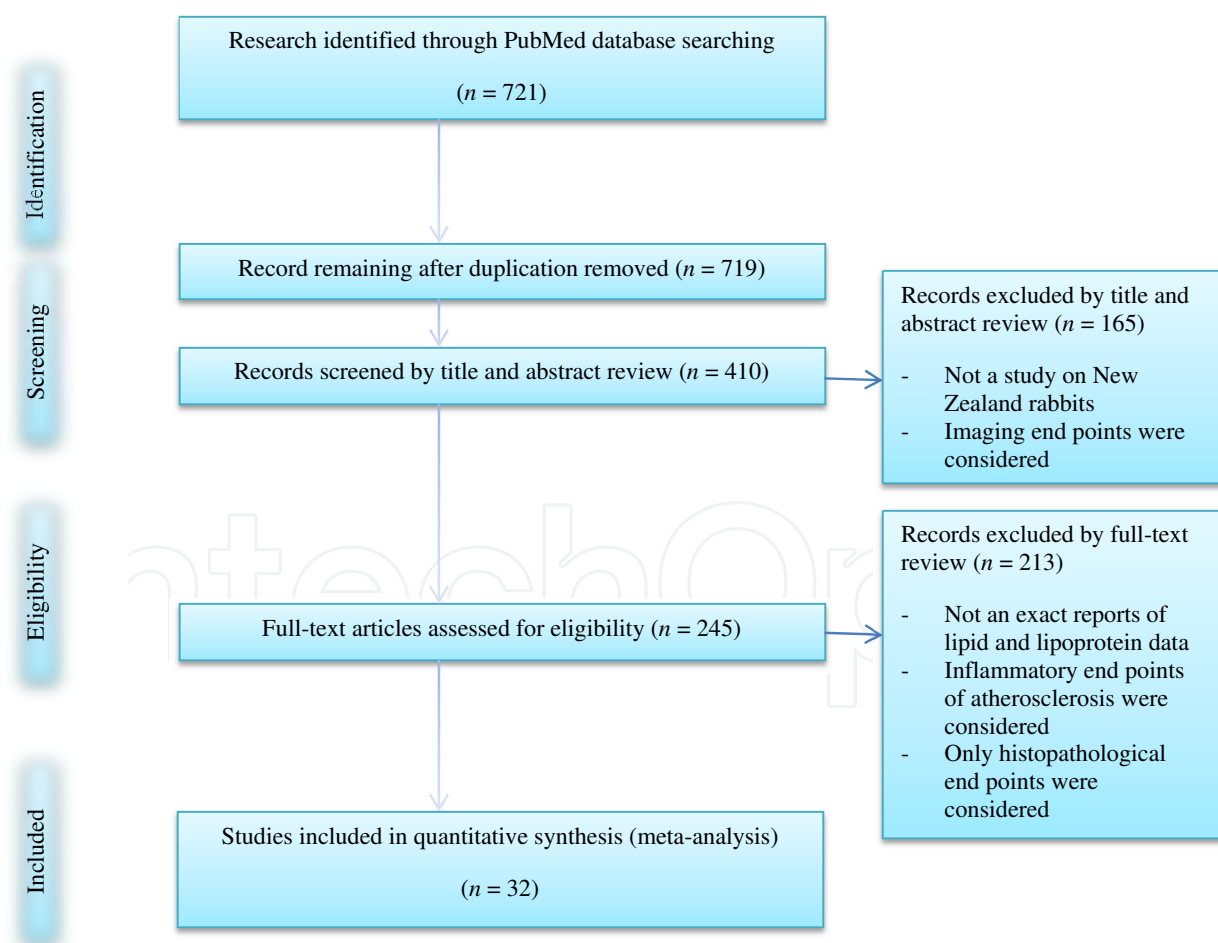


Figure 1. Flow diagram of publication inclusion.

2.2. Statistical analysis

Effect sizes are indices that measure the magnitude of the differences between two groups. For each comparison, individual RCT data for each outcome measure and combined measure were pooled to calculate standardized mean difference (SMD) effect size considering $P < 0.05$ significant level [42] using the Comprehensive Meta-Analysis ver.2.2.064, a software package developed by Biostat (<http://www.meta-analysis.com/>; Englewood, NJ 08631 USA).

To show substantial heterogeneity among studies, we report fixed-, random-, and mixed-effects meta-analysis [43]. In this continuum, random-effects meta-analysis takes into account the precision of discrete studies and the variation among studies and weights of each study accordingly.

We conducted two subgroup analyses to explore association of dietary cholesterol and duration of cholesterol intake with outcome measures. For each subgroup category, overall net change estimates were calculated using fixed-effects, random-effects, and mixed-effects models, and the heterogeneity of estimates was assessed. We conducted a meta-regression analysis to further examine the effects of cholesterol intake as an explanatory factor on outcome variables using random-effects meta-regression (unrestricted maximum likelihood (UREML)).

The heterogeneity of studies was quantified using chi-square test, Q , and I^2 statistics [44]. Begg's [45] and Egger's tests [46] were employed to identify publication bias.

3. Results and discussion

A total of 721 articles were initially identified after electronic search from January 1, 2004, to January 1, 2015, was restricted to animal studies using usual PubMed filters. Three duplicated articles were subtracted, and 719 unique publications were screened by title and abstract review. In this continuum, articles dealing with non-New Zealand rabbit strains and studying imaging end points like MRI, CT scan, and radiology have been excluded (see Figure 1). The remaining articles were submitted to detail inspection, and articles that focused on inflammatory and histopathologic end points of atherosclerosis without reporting lipid and lipoprotein profiles of studied animals were excluded. In total, 32 eligible studies were included in the systematic review and meta-analysis involving 1104 rabbit subjects [10–41]. We just considered control and cholesterol-fed (treated; model) rabbits for meta-analytic assessment, and other animal groups that received other interventions (drug, nutrients, etc.) have been excluded from the data set. The study identification process is shown in Figure 1.

Of the included RCT, 30 studies reported on outcomes of triacylglycerols (TGs; $n = 638$ animal subjects), 32 studies reported on total cholesterol (TC; $n = 677$ animal subjects), 26 studies reported on LDL-C ($n = 595$ animal subjects), 30 studies reported on high-density lipoprotein cholesterol (HDL-C; $n = 571$ animal subjects), 4 studies reported on LDL-C/HDL-C ($n = 98$ animal subjects), 1 study reported on LDL-C/TC ($n = 8$ animal subjects), 2 studies reported on oxidized LDL-C (ox-LDL-C; $n = 32$ animal subjects), and 3 studies reported on TC/HDL-C ($n = 46$ animal subjects).

Age in the start of studies varied from 1.86 to 9 months old with an average value of 4.37 ± 2.52 months old in 9 studies, and it was not reported in the majority of studies. The duration of nutritional intervention went from 4 to 56 weeks with an average value of 8.90 ± 7.26 weeks in all studies (Figure 2). The majority of studies (56%) reported ≤ 10 months, while 37.5% of studies reported 10–20 weeks of nutritional intervention to translate a rabbit model of atherosclerosis. The amount of dietary cholesterol surplus that utilized to induce atherosclerosis varied from 0.34% to 4.00% with an average value of $0.98 \pm 0.67\%$ in all studies (Figure 3). In this context, 31% of all eligible studies used 0.5% cholesterol in feed, while 46% of studies used 1% cholesterol in their feed.

The approximate analysis and the major ingredients of rations were not usually reported in studies. However, in 40.6% of studies, a range of fat (1% to 10%) with an average value of ($5.03 \pm 2.80\%$) from diverse sources like lard has been reported. The 5% fat content has been more frequently (30.76%) utilized among studies that reported the fat content of diets. However, it is not precisely clear from studies that the fat added to the ration is the whole content of dietary fat or surplus fat. Experimental diets must be formulated according to the rabbit requirements of NRC [47] because all macronutrients (carbohydrate, fat, and protein), micronutrients (minerals and vitamins), and other diet-specific bioactive compounds may modulate lipid metabolism and cytokine milieu that lead to atherosclerosis or prevent atherosclerosis. In addition, the cholesterol content of diets needs to be determined since 0.34% cholesterol in diet can trigger dyslipidemia in rabbits, depending on the duration of treatment. The lack of dietary formulations and the approximate analysis are two major shortcomings of studies that report cholesterol-induced atherosclerosis in rabbits.

No consensus for initial weights of rabbits were observed among eligible studies as well as on the changes of weight during experiment and the final weight of the studied animal at the end of dietary intervention. The initial weight of rabbits employed to translate atherosclerosis changed from 1.5 to 3.25 kg with an average value of 2.45 ± 0.59 kg. Final weights (3.29 ± 0.21 kg) were reported only in four studies, in which their rabbits were fed 1% cholesterol for an average duration of 2.17 ± 0.84 months of nutritional intervention. By pooling data from 32 RCTs of 1104 rabbit subjects, the current meta-analysis documented lipid and lipoprotein alterations associated with increased intake of cholesterol. However, dietary formulations, duration of cholesterol feeding, initial and final weights of animals or their weight changes during study, and concise age of animals were not reported in most of these studies and would cause heterogeneity.

The effect of dietary cholesterol inclusion on the combined outcome of lipid and lipoprotein profiles and indices of rabbits in a random-effect model as an analysis model for the meta-analyses was 5.618 (95% confidence interval (CI): 4.592, 6.644; $P = 0.0001$). In this way, SMD effects of dietary cholesterol inclusion were 2.424 mg/dl (95% CI: 1.531, 3.318 mg/dl; $P = 0.000009$) for HDL-C, 7.646 mg/dl (6.266, 9.026; $P = 0.000000000$) for LDL-C, 5.216 (95% CI: 2.155, 8.278; $P = 0.001$) for LDL-C/HDL-C, 4.658 ng/ml (95% CI: 3.321, 5.995 ng/ml; $P = 0.000000000$) for ox-LDL-C, 8.643 mg/dl (95% CI: 7.258, 10.028 mg/dl; $P = 0.000000000$) for TC, 4.392 (95% CI: 2.317, 6.466; $P = 0.00003$) for TC/HDL-C, and 3.173 mg/dl (95% CI: 2.451, 3.896 mg/dl; $P = 0.000000000$) for TGs. The value of I^2 was 89.387%, indicating that nearly all of the

variation is real and sturdily supporting the subgroup analysis and/or the meta-regression. By convention, an I^2 value $>75\%$ indicates significant between-study heterogeneity [4]. Subgroup analyses according to whether lipid and lipoprotein changes occurred following the stratification of studies according to the duration of cholesterol feeding in cholesterol-fed rabbits. In this way, based on a mixed-effects analysis model, SMD effects of the duration of cholesterol inclusion was 2.788 (95% CI: 2.333, 3.244; $P = 0.000009$). According to the Q test ($Q = 112.206$, $P < 0.001$), a significant level of heterogeneity has been concluded in the subgroup analysis of studies based on the duration of cholesterol feeding, which reflects the diversity of study design among the 28 comparisons.

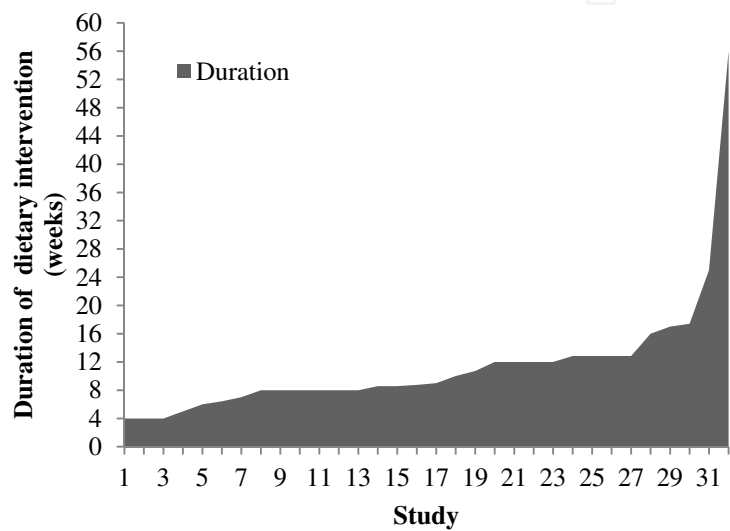


Figure 2. The duration of dietary cholesterol surplus used in a rabbit model of atherosclerosis.

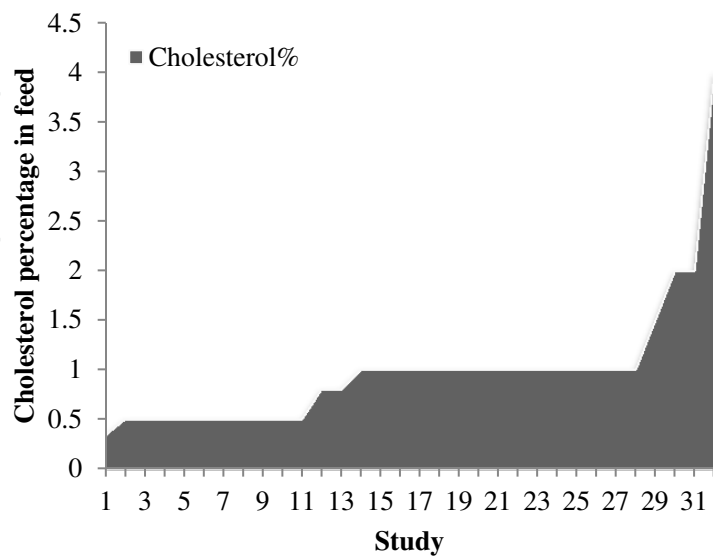


Figure 3. The dietary cholesterol surplus used in a rabbit model of atherosclerosis.

In this continuum, the subgroup analysis based on duration (week) of cholesterol feeding in a mixed-effects analysis showed combined SMD effects, that is, 2.788 (95% CI: 2.333, 3.244; $P = 0.000$; $Q = 112.206$; $df = 14$). Furthermore, SMD effects were 1.414 mg/dl (95% CI: 1.049, 1.780; $P = 0.000000000$; $Q = 166.780$; $df = 14$) for HDL-C, 4.3888 mg/dl (95% CI: 3.779, 4.996; $P = 0.000000000$; $Q = 138.362$; $df = 13$) for LDL-C, 3.367 (95% CI: 2.686, 4.048; $P = 0.000000$; $Q = 35.845$; $df = 3$) for LDL-C/HDL-C, 4.658 ng/ml (95% CI: 3.321, 5.995 ng/ml; $P = 0.000000000$; $Q = 0.163$; $df = 1$) for ox-LDL-C, 3.942 mg/dl (95% CI: 3.408, 4.475 mg/dl; $P = 0.000000000$; $Q = 184.421$; $df = 17$) for TC, 3.725 (95% CI: 2.733, 4.718; $P = 0.000$; $Q = 6.663$; $df = 2$) for TC/HDL-C, and 2.161 mg/dl (95% CI: 1.792, 2.529 mg/dl; $P = 0.000000000$; $Q = 79.221$; $df = 15$) for TGs. The duration of cholesterol intake was an important determinant of the heterogeneity in studies with regard to lipid and lipoprotein outcomes. Based on Q values of outcomes following the subgroup analysis and their comparison with the chi-square table, the high level of heterogeneity has been detected for all outcomes except TC/HDL-C as an atherogenic index. This strange result may be due to the low sample size ($n = 3$) of the TC/HDL-C index. Therefore, the duration of cholesterol intake may not play an essential role in inducing dyslipidemic atherogenesis in cholesterol-fed rabbit models.

Subgroup analyses according to whether lipid and lipoprotein changes occurred following the stratification of studies according to the amount of cholesterol feeding in cholesterol-fed rabbits. In this continuum, the subgroup analysis based on dietary cholesterol inclusion (%) in of cholesterol-fed rabbits in a mixed-effects analysis showed SMD effects 5.538 (95% CI: 4.613, 6.463; $P = 0.000$; $Q = 31.622$; $df = 6$). Further, the subgroup analysis based on the amount of cholesterol feeding in a mixed-effects analysis showed SMD effects 1.743 mg/dl (95% CI: 1.145, 2.341; $P = 0.000000000$; $Q = 77.709$; $df = 6$) for HDL-C, 7.409 mg/dl (95% CI: 6.173, 8.646; $P = 0.000000000$; $Q = 138.362$; $df = 4$) for LDL-C, 3.208 (95% CI: 1.680, 4.736; $P = 0.000000000$; $Q = 0.967$; $df = 1$) for LDL-C/HDL-C, 4.658 ng/ml (95% CI: 3.321, 5.995 ng/ml; $P = 0.000000000$; $Q =$ not determined; $df = 0$) for ox-LDL-C, 8.543 mg/dl (95% CI: 7.309, 9.776 mg/dl; $P = 0.000000000$; $Q = 41.307$; $df = 6$) for TC, 4.255 (95% CI: 2.852, 5.658; $P = 0.000$; $Q = 3.691$; $df = 1$) for TC/HDL-C, and 3.107 mg/dl (95% CI: 2.491, 3.723 mg/dl; $P = 0.000000000$; $Q = 23.675$; $df = 6$) for TGs.

As the amount of dietary cholesterol surplus was considered as a moderator, the P values of Q_R in mixed-model regression were significant for TGs, TC, LDL-C/HDL-C, LDL-C, HDL-C, and combined lipid and lipid profile ($P < 0.01$; Table 1 and Figure 4), while the P values of Q_R in mixed-model regression were nonsignificant for TC/HDL-C ($P = 0.31731$; Table 1). Random-effects meta-regression (UREML) was conducted for change in all parameters and indices using the cholesterol moderator (data not shown). The ANOVA table of fixed- and mixed-effect models for combined lipid and lipoprotein profile regression is shown in Table 1. Accordingly, Q_M is significant. Then at least one of the regression coefficients between dietary cholesterol inclusion and lipid and lipoprotein profiles and indices is different from zero. In this sense, the funnel plot (Figure 5) and the results of Egger's tests showed a significant publication bias (data not shown). The subgroup analysis based on the amount of dietary cholesterol surplus showed heterogeneity in all outcomes except LDL-C/HDL-C ($n = 2$) and TC/HDL-C ($n = 2$) after comparing their Q values using the chi-square table. Therefore, cholesterol content may not be a pivotal determinant in inducing dyslipidemia in cholesterol-

fed rabbit models, but it raised all lipid and lipoprotein profile and indices in cholesterol-fed rabbits. Moreover, no consensus for atherogenic diets was observed based on their cholesterol and fat contents and the types of their fat, including pure cholesterol, lard, egg yolk powder, coconut oil, and/or safflower oil as well as different nutritional interventions.

ANOVA						
Fixed-effect regression				Mixed effects regression		
	<i>Q</i>	df	<i>P</i>	<i>Q</i>	df	<i>P</i>
<i>Q_M</i>	44.51969	1	0.00000	5.30338	1	0.02128
<i>Q_R</i>	287.66695	34	0.00000	43.61079	34	0.12506
<i>Q_{TOTAL}</i>	332.18664	35	0.00000	48.91418	35	0.05934

Q_M—model sum of squares compared to chi-square distribution with *p*– 1 df (*p* is number of predictors in the model); *Q_R*—residual sum of squares compared to chi-square distribution with *k*– *p*– 1 df (*k* is the number of studies) [48].

Table 1. Fixed- and mixed-effect models—analysis of variance (ANOVA) table for combined lipid and lipoprotein profile regression.

The resulting funnel graphs and the associated statistics based on Egger et al. [46] revealed a significant asymmetry and publication bias among studies. Therefore, publication or other sources of bias could be relevant in evaluating an overall effect of the selected nutritional interventions in lipid and lipoprotein outcomes in the cholesterol-fed rabbit model of (pre)atherosclerosis.

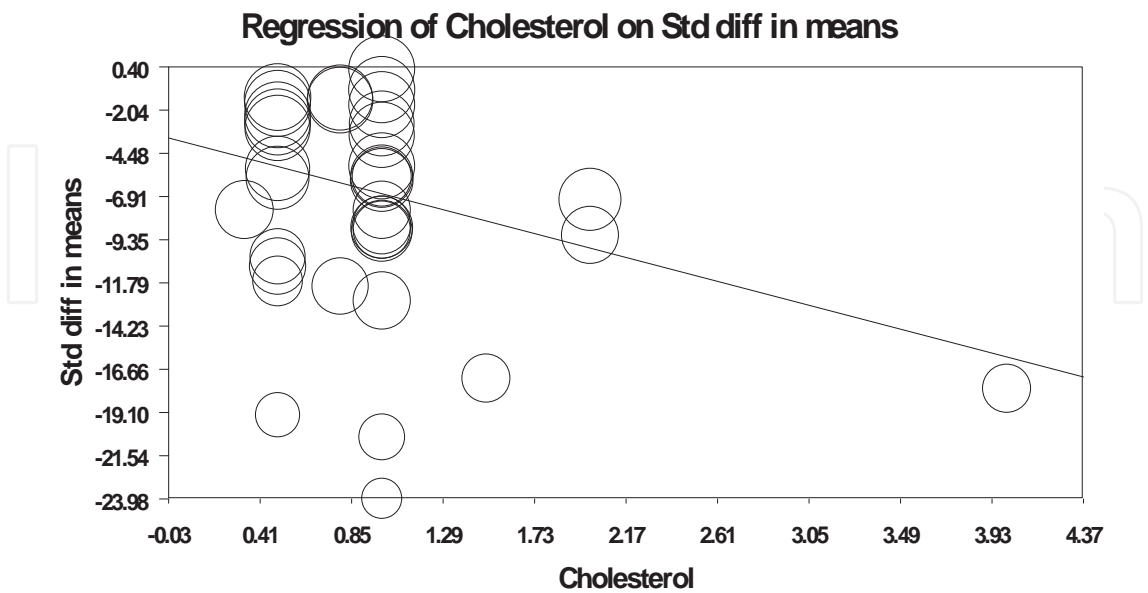


Figure 4. Fixed-effect model—regression of dietary cholesterol surplus on standardized mean difference of lipid and lipid profiles and indices in a rabbit model of atherosclerosis.



Figure 5. Funnel plot for a meta-analysis of association between dietary cholesterol inclusion and combined effects of lipid and lipoprotein profiles and indices in a rabbit model of atherosclerosis.

4. Conclusions

These systematic review and meta-analyses of RCTs initially assess the robustness of cholesterol-fed rabbit of atherosclerosis since the causal role of cholesterol intake in progression and induction of atherosclerosis is not supported in some clinical trials (e.g., see [49]). These systematic review and meta-analysis showed high levels of heterogeneity among studies that used the cholesterol-fed rabbit model of atherosclerosis, which may be due to the lack of consensus on the dietary ingredients and formulation, the amount of dietary cholesterol surplus, the duration of cholesterol intake, and the analytical methodology in lipid and lipoprotein determination. In sum, for the first time, this meta-analysis showed that dietary cholesterol surplus could not be a reliable determinant of dyslipidemic atherosclerosis in cholesterol-fed rabbits. Rabbits in fact are vegetarians, and plants actually do not contain cholesterol. Thus, by itself, the model for studying atherogenesis by cholesterol feeding is far from being ideal or relevant for the situation in humans.

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